A ROLE OF INTRACELLULAR POLYSACCHARIDE IN THE SPORULATION OF *BACILLUS CEREUS*

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by

Steven MacKay Schmid

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by
Steven MacKay Schmid

Approved by Committee:

Dean G. Hoganson
Chairperson

Michael E. Mygrecoli

Lawrence L. Burm

Earle L. Canfield
Dean of the School of Graduate Studies

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An abstract of a Thesis by
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The problem. *Bacillus cereus* accumulates an intracellular polysaccharide under conditions of nutritional stress. The polysaccharide is then degraded as the organism begins sporulation. This study concerns intracellular polysaccharide and its possible involvement in sporulation as a source of carbon and energy.

Procedures. Ethyl methanesulphonate mutagenized cells of *Bacillus cereus* 10702 were screened for the ability to accumulate glucose during growth with glucostat reagent. Glucose deficient mutants were assayed for the ability to accumulate poly-β-hydroxybutyric acid and intracellular polysaccharide followed by comparison to wild type levels. Endotrophic sporulation capabilities with exogenous glucose were determined by the heat resistance of spores in both mutant and wild type. Dipicolinic acid levels in spores were assayed with modified Janssen reagent.

Findings. A mutant of *Bacillus cereus* strain T isolated was deficient in the ability to accumulate intracellular polysaccharide. The mutant produced low levels of heat sensitive spores. Heat sensitivity was dependent upon poly-β-hydroxybutyric acid accumulation and levels of exogenous glucose during sporulation. Inhibition of poly-β-hydroxybutyric acid accumulation increased heat resistance of spores subjected to sporulation in low levels of glucose and $CaCl_2$.

Conclusions. The data indicated that intracellular polysaccharide supplied the sporulation process with a carbon and/or energy source involved in spore formation and heat resistance.

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INTRODUCTION

A wide variety of bacteria have the ability to accumulate and degrade an intracellular polysaccharide during the log and stationary phases of growth. The accumulation was greatest under conditions when the media lacked a nutrient factor which was not needed for intracellular polysaccharide synthesis (Wilkinson, 1959). Work conducted with Escherichia coli revealed the presence of glycogen granules by a comparison of "holes" in the cytoplasm and glycogen content of the cells during growth. The maximum amount of glycogen accumulation was noted in cells grown in nitrogen deficient media. A later addition of the limiting nutrient resulted in a marked decrease in glycogen content with a corresponding increase to total cell mass (Holme and Cedergren, 1961). In Clostridium botulinum type D' an intracellular polysaccharide was accumulated during late log and early stationary phase and then was used as an endogenous carbon and energy source for sporulation (Strasdine, 1972). The compound must fulfill three criteria to be considered an internal carbon and energy source (Wilkinson, 1959). There must be an accumulation of the compound when exogenous nutrient sources were greater than needed for growth. The compound must be

used as the exogenous nutrient sources fall below levels necessary for growth. The compound must have a role in the organism's ability to survive. The above organisms have been shown to synthesize an endogenous carbon and energy compound for use in periods of nutritive stress. A possible role of an intracellular polysaccharide in sporogenesis has been proposed, and the following paper will be concerned with this area.

In a fine structure study of Bacillus cereus, areas resembling glycogen particles were found in the cell. These particles appeared during the late log phase and declined during sporulation. Location of the granules during stationary phase were along the cell membrane. As sporulation proceeded, the glycogen like particles slowly dispersed (Ellar and Lundgren, 1966). The existence of an intracellular polysaccharide was demonstrated in Bacillus cereus strain T (Slock and Stahly, 1974). A partial characterization of the polysaccharide indicated that the molecule contained glucose. The polysaccharide has a proposed structure similar to amylopectin containing both $\alpha 1 \longrightarrow 6$ and $\alpha 1 \longrightarrow 4$ linkages. During growth in a glucose limited media the polysaccharide accumulated until exogenous supplies of glucose were exhausted. Degradation of the polysaccharide occurred during early spore formation. The same behavior was observed in nitrogen limited media except the level of intracellular polysaccharide

accumulation was greater. The characteristics of this polysaccharide partially fulfill the criteria for an endogenous carbon and energy source for Bacillus cereus. The object of this study was to analyze the polysaccharide in terms of its role in the organism's ability to survive through sporogenesis.

A compound already known to be an internal carbon and energy source is poly-β-hydroxybutyric acid (PHB) which is characterized as a lipid storage compound (Slepecky and Law, 1961). In Bacillus cereus PHB accumulates during late log phase and is degraded during the stationary and sporulation phases. A study with Bacillus cereus and C^{14} labeled acetate demonstrated that acetate in the media was taken up by the cells and incorporated into PHB (Nakata, 1966). The labeled PHB was later degraded into labeled intermediates which were incorporated into dipicolinic acid (DPA), spore proteins, and CO_2. The data indicated that PHB was a carbon and energy storage compound. The PHB appeared in the cells as large granules or vacuoles (Ellar and Lundgren, 1966). PHB did not accumulate in significant amounts when growth occurred in media that was strongly buffered at pH = 7.4 (Kominek, 1964). Although little PHB was formed when the media was buffered at 7.4, the sporulation capabilities of the cells were not impaired and fully resistant spores resulted. Cells with low levels

of PHB also produced equally resistant spores when allowed to sporulate endotrophically. From this evidence it was concluded that PHB was not an essential internal carbon and energy source for Bacillus cereus.

The intracellular polysaccharide displayed similar characteristics to a polysaccharide that appeared to be attached to the cell wall of Bacillus cereus. Upon sonic breakage of the cells a polysaccharide was found tightly bound to the cell wall (Slock and Stahly, 1974). During growth, the cell wall-attached polysaccharide level closely correlated the accumulation and degradation levels of the intracellular polysaccharide. Further study showed that the two polymers apparently had the same structure. A procedure employing 10% trichloroacetic acid extraction and ethanol precipitation removed all cell wall and intracellular polysaccharides from cell debris. Electron micrographs of B. cereus indicated that glycogen like areas were located along the cell membrane (Ellar and Lundgren, 1966). The cell wall polysaccharide may be a part of the intracellular polysaccharide vacuole bound to the cell wall, which remained bound on the cell wall upon sonic disruption of the cell. Other tests indicated that glucose was found primarily in polysaccharide form and no free glucose could be detected (Slock and Stahly, 1974).

In this study mutant strains deficient in the ability to accumulate the intracellular polysaccharide were

isolated and compared in characteristics of sporulation to the wild type. The intracellular polysaccharide was found to be required for the process of sporulation since spores produced by the mutant did not show the same resistance to heat as the wild type spores. A study of the effects of glucose concentration during sporulation was undertaken in an effort to gain insight into the nature of the intracellular polysaccharide requirement.

MATERIALS AND METHODS

Organisms studied. Bacillus cereus strain T was used as the test organism along with selected mutant strains Bacillus cereus strain T that were exposed to ethylmethanesulfonate.

Storage and cultivation of the organisms. Spore stocks of the mutant strains and wild type Bacillus cereus were maintained at 3°C on nutrient agar slants and in distilled water at -20°C.

Cultivation of the organism was begun with a $1:1x10^6$ dilution of the spore stock followed by growth in a starter culture at 31°C on a shaker machine or shaker bath (Eberbach Co.). When the cells reached a state of exponential growth a 10% transfer was made into fresh media. The use of the transfer enhanced the homogenicity of the cell population.

Media for growth and sporulation. G-Medium. The medium was developed to support growth and sporulation (Greenburg, 1954). In G-medium free spores were produced within 24 hours of innoculation with exponential phase cells.

Separate stock solutions of 10% glucose, 5% K_2HPO_4, 0.8% $CaCl_2$, and 10% yeast extract were made with distilled

water and autoclaved. A mineral solution was prepared with 0.01% $FeSO_4 \cdot 7H_2O$, 0.1% $CuSO_4 \cdot 5H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$, 1.0% $MnSO_4 \cdot H_2O$, and 4.0% $MgSO_4$ in distilled water and then combined with an equal volume of 40% $(NH_4)_2SO_4$ in distilled water and stored at room temperature.

The medium was prepared by adding mineral solution to a preset volume of distilled water to give a final volume concentration of 1.0%. This solution was autoclaved and cooled to room temperature. Stock solutions of glucose, potassium phosphate, calcium chloride, and yeast extract were added aseptically to give final concentrations of 1.0, 1.0, 1.0, and 2.0% respectively. The pH of G-media once prepared was 7.2-7.4.

N-Medium. N-medium was developed to enhance polysaccharide production (Slock, 1970). It is prepared in the same manner as G-medium except for the following changes: final glucose concentration was raised to 2.0%, and final yeast extract concentration was dropped to 1.0%.

N and G-medium buffered to 7.4. These two media were prepared in the same manner as N and G medium but 5.0% K_2HPO_4 was replaced with potassium phosphate buffer to give a final concentration of 1.0 molar and a pH of 7.4.

Assay of total glucose content. Samples of 100 ml were taken from growing cultures and washed twice with distilled water at 4°C. The pellets were suspended in 5.0 ml of 2N H_2SO_4 and hydrolysed in a boiling water bath for

3 hours. The samples were centrifuged and washed with 5.0 ml. of distilled water. The original and wash supernatants were combined. The samples were then neutralized with 2M K_2HPO_4 to a pH of 7.0. Glucose content was assayed using glucostat reagent (Worthington Co.) using the macro method described by the manufacturer. The reaction was allowed to go to completion in 2 hours at 37°C.

Assay for intracellular polysaccharide content. Culture samples of one hundred ml. were taken at regular intervals during growth. The cells were centrifuged and washed twice with distilled water at 4°C. After resuspending the pellets in 20.0 ml. H_2O, the cells were disrupted by sonication at 50 watts with a Heat Systems, Inc. sonifier, model W-185 for five minutes at 4°C. Examination of cells was done with a phase contrast microscope to assure complete disruption. The samples were then centrifuged and supernatants were separated from cell wall fractions. Trichloroacetic acid (TCA) was added to the supernatant to give a final concentration of 2.5%. The samples were mixed and incubated at 4°C for 10 minutes to precipitate out protein. After centrifugation the supernatants were decanted. A 5 ml. wash of 2.5% TCA was added to the pellets and after centrifugation the supernatants were combined. Sample volume was decreased at 70°C in a current of air. A saturated solution of Na_2SO_4 added at

the rate of 0.1 ml/ml. of sample volume was followed by the addition of 1.3 volumes of ethanol to precipitate out the polysaccharide. The precipitate was centrifuged and the supernatant discarded. The polysaccharide was dissolved in 2 ml. H_2O and reprecipitated with 1.3 volumes of ethanol, centrifuged and the supernatants discarded. The samples were then treated with amylglucosidase (Sigma Co.) and followed with glucostat special reagent. The absorbance of the samples was read at 420 nm and values were obtained by plotting against a prepared standard curve of known values.

Assay of poly-β-hydroxybutyric acid content in cells. Determination of poly-β-hydroxybutyric acid (PHB) involved the isolation and conversion of PHB to crotonic acid in concentrated H_2SO_4 (Law and Slepecky, 1961). Samples of ten ml. were taken from growing cultures and centrifuged. The supernatant was discarded and the cells were resuspended in 10.0 ml. of 6% sodium hypochlorite. After incubation at 37°C for 1 hour in a water bath, the samples were centrifuged and the supernatants discarded. The samples were then washed with water, acetone, and ethanol all near their boiling points. The pellets were fully suspended in each wash, spun down, and all supernatants were discarded. Extraction of PHB was done with three 2.5 ml. portions of boiling chloroform. The samples were centrifuged and the supernatants combined. The samples were passed through a

fritted glass filter with a 2.5 ml. portion of chloroform added to clear the filter. The chloroform was evaporated off in an air current at 55°C. Volumes of twenty ml. concentrated H_2SO_4 were added and sample tubes were capped with a marble. The samples were refluxed at 100°C for 10 minutes and then cooled to room temperature. Absorbance of the samples was read at 235 nm against a H_2SO_4 blank. Concentration was calculated using the molarity extinction coefficient of crotonic acid = 1.56×10^4.

Heat resistance study of endotrophically produced spores. At various intervals during growth, samples were taken, centrifuged, washed twice with distilled water and placed in sterile 0.008% $CaCl_2$ solution. The samples were incubated for 24 hours at 31°C in a shaker machine so that endotrophic sporulation could take place. Samples were diluted to 10^{-7} and 10^{-8} and 0.1 ml. of each was plated on nutrient agar plates. The dilutions were then subjected to 80°C in a waterbath. At various times samples were drawn off and plated on nutrient agar. After 24 hours at room temperature the plates were counted with a Quebec colony counter.

Heat resistance study with glucose added to endosporulation media. This procedure involved a heat resistance study with the exception that samples taken were allowed to sporulate in solutions containing 0.008% $CaCl_2$ with 1.0 and 0.1% glucose.

Dipicolinic acid assay with modified Janssen reagent. The modified Janssen color reagent contains 1% $Fe(NH_4)_2SO_4 \cdot 6H_2O$ and 0.1% cysteine dissolved in 0.05 molar acetate buffer at a pH = 4.0. The assay involved taking a 2.0 ml. sample of the culture, centrifuging, and discarding the supernatant. Six ml. of H_2O was added and the samples were autoclaved for 15 minutes. After cooling the samples to room temperature, 0.1 ml. of 1N acetic acid was added to acidify the samples. The samples were incubated for one hour and centrifuged. Four ml. of supernatant from each sample was pipetted into a clean test tube. One ml. of color reagent was added to each supernatant. Sample absorbance was read at 440 nm with a blank of 4.0 ml. of sample and 1.0 ml. H_2O. Values were obtained by comparing absorbance of the samples against a prepared standard curve of known values (Janssen, 1958).

Observation and direct counts of the organism. The organism was observed during growth with a phase contrast microscope (American Optical Inc.). Direct bacterial counts were made with the use of a Petroff Hauser bacterial counting chamber.

Centrifugation. Centrifugation of samples was performed with a Lourdes Betafuge with a fixed angle head and an IEC clinical centrifuge with fixed angle head.

Absorbance, optical density, and pH measurements. The absorbance of cultures were measured at 595 nm on a

Bausch and Lomb Spectronic 20 spectrophotometer. Measurements of the absorbance from the various assays were made with a dual beam spectrophotometer with visible and ultraviolet light sources (Beckman Instruments, Inc. Model ACTA III). The pH of samples was determined with a Corning Model 10 pH meter.

RESULTS

Study of wild type growth. Growth characteristics of B. cereus was used for comparison against selected mutant strains deficient in intracellular polysaccharide (Fig. 1 and Table 1).

G medium. During vegetative growth a drop in pH occurred, followed by an increase during the stationary and sporulating phases. This behavior was consistent with previous studies (Nakata and Halverson, 1960). The drop and subsequent rise of pH were due to the oxidation of glucose to acetate and pyruvate followed by their utilization in oxidative phosphorylation and biosynthetic pathways (Nakata and Halverson, 1960). Total glucose content increased during vegetative growth and decreased as the cells entered the stationary phase at 4 hr. A second build up of glucose occurred in the stationary phase along with the appearance of storage vacuoles. Earlier studies indicated intermediate acid utilization coincided with the exhaustion of exogenous glucose (Nakata and Halverson, 1960). The glucose content of cells also coincided with acid utilization and this suggested that glucose was synthesized by gluconeogenesis past 4 hr. The glucose content declined with the appearance of forespores at 7 hr. The spores

TABLE 1

GROWTH CURVE OF BACILLUS CEREUS

Time	Absorbance at 595 nm	pH	Cell Morphology
		in G media	
0.0	0.01	6.85	long chains, even cytoplasm
0.5	0.02	6.86	
1.0	0.07	6.72	
1.5	0.17	6.34	
2.0	0.44	5.89	
2.5	0.53	5.05	cell wall more distinct, cells shortening
3.0	0.63	4.90	
3.5	0.75	4.80	
4.0	0.80	5.00	
4.5	0.82	5.40	appearance of vacuoles
5.0	0.85	5.65	single cells and chains
5.5	0.99	6.30	
6.0	1.20	6.74	
6.5	1.10	6.95	
7.0	1.20	7.10	
7.5	1.10	7.20	
8.0	1.10	7.30	appearance of forespores
8.5	1.20	7.40	
9.0	1.30	7.40	
9.5	1.30	7.40	
10.0	1.40	7.54	short chains and single cells
10.5	1.40	7.64	
11.0	1.45	7.60	
11.5	1.45	7.67	refractile spores inside the cells
12.0	1.45	7.73	
12.5	1.45	7.77	
13.0	1.45	7.73	
		in N media	
0.0	0.01	7.20	long chains, even cytoplasm
0.5	0.01	7.19	
1.0	0.05	7.20	
1.5	0.14	7.07	chains, two cells, and motile single cells

TABLE 1--Continued

Time	Absorbance at 595 nm	pH	Cell Morphology
2.0	0.29	6.76	
2.5	0.49	6.45	appearance of vacuoles
3.0	0.59	6.46	cells shortening
3.5	0.66	6.56	
4.0	0.75	6.58	
4.5	0.80	6.60	
5.0	0.85	6.65	appearance of forespores
5.5	0.87	6.68	
6.0	0.93	6.65	
6.5	0.93	6.75	
7.0	1.00	6.70	
7.5	1.25	6.80	
8.0	1.30	6.64	
8.5	1.40	6.64	
9.0	1.45	6.63	refractile spores inside the cells
9.5	1.45	6.59	
10.0	1.45	6.57	
10.5	1.45	6.59	
11.0	1.45	6.58	
11.5	1.45	6.53	

Figure 1

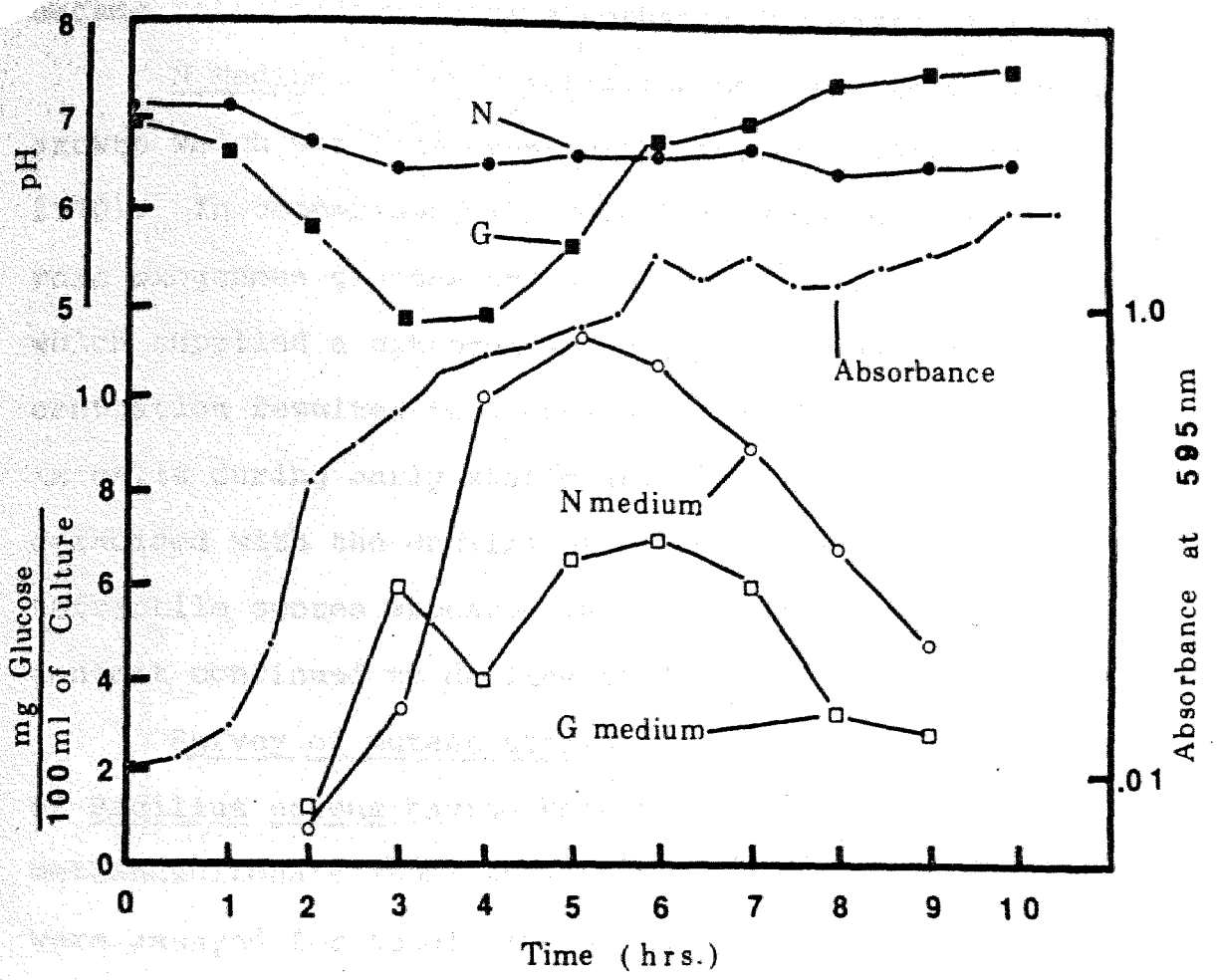

Figure 1. Growth and total glucose content of Bacillus cereus in weakly buffered G and N medium. Cells were inoculated and grown according to procedures in materials and methods. Total glucose was assayed with glucostat reagent as described in materials and methods. Total mg glucose in cells is represented in G medium (□) and N medium (o). The pH of culture medium G (■) and N (●) is shown in the upper scale.

became refractile and the absorbance increased at 11 hr.

N medium. Little variation occurred in pH during growth which was consistent with earlier studies (Slock, 1970). In comparison with G medium, N medium contained more exogenous glucose and was limited in yeast extract which supplied a nitrogen source. The higher glucose concentration resulted in a greater accumulation of glucose in cells during early stationary phase. Forespore formation coincided with the decline of glucose content at 5 hr. Refractile spores appeared in the cells as the glucose content continued to decline at 9 hr.

Survey of mutant strains in G and N medium. Strains of Bacillus cereus having been mutagenised with ethyl methanesulfonate (EMS) were grown in G and N medium and were assayed for total glucose content at various stages of development. During the growth phases cells were examined with phase contrast microscopy to screen out mutants with major defects in morphology. Growth curves of the mutants grown in G medium and N medium are shown on Figures 2 and 3 respectively. Only strains which showed the most favorable characteristics were presented. Of the mutants shown, dglc 9 (deficient in glucose content) showed the lowest levels of glucose content in both G and N medium. Microscopic examination of dglc 9 at various stages of growth revealed that the cells were of irregular shapes and autolysis occurred in early stationary phase. No mature

Figure 2

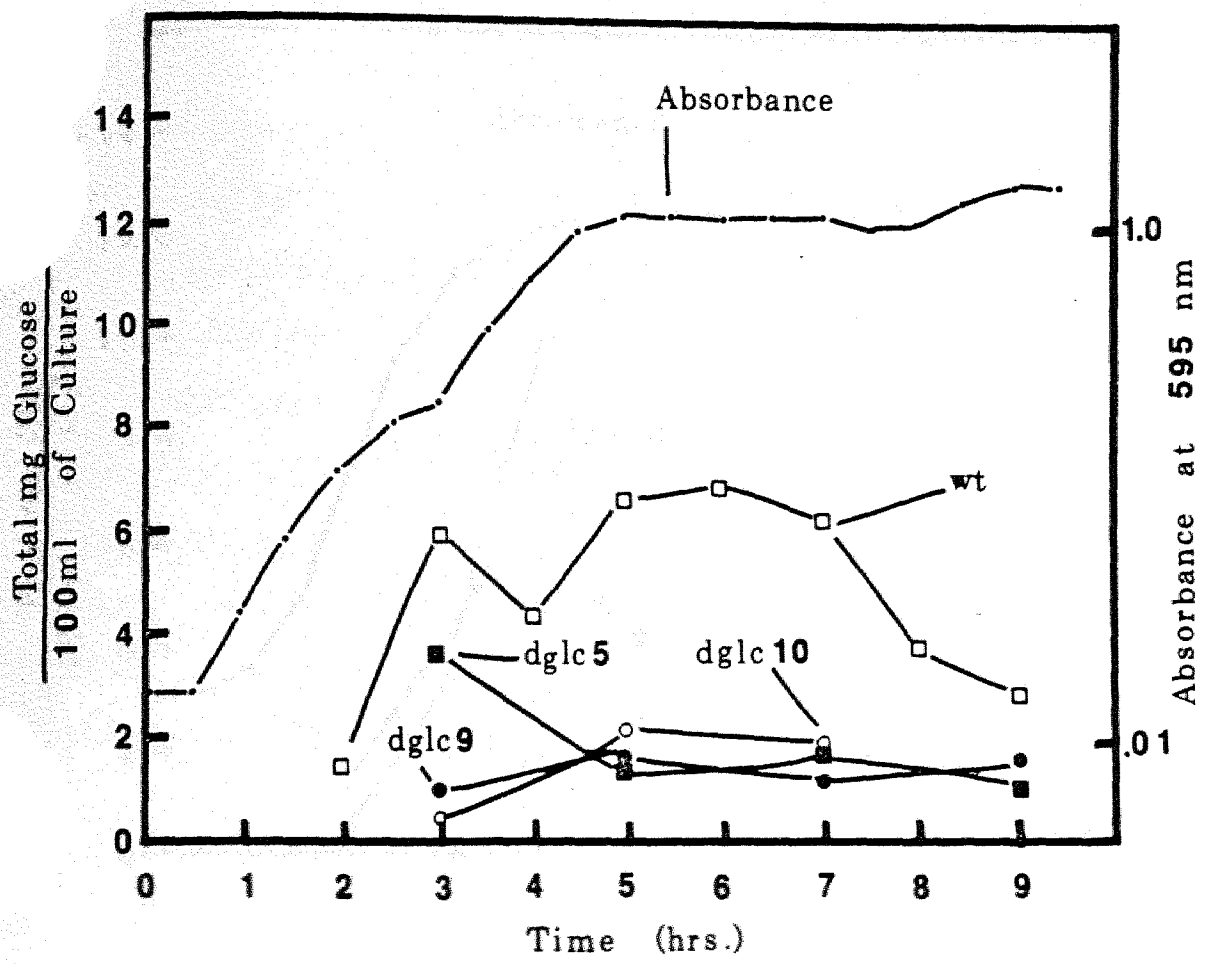

Figure 2. Total glucose of mutant cells in weakly buffered G medium. Mutant types 5, 9, and 10 were grown in G medium and total glucose was measured with glucostat reagent as described in materials and methods. Measurements of total glucose in the wild type organism grown in weakly buffered G medium are shown for comparison.

Figure 3

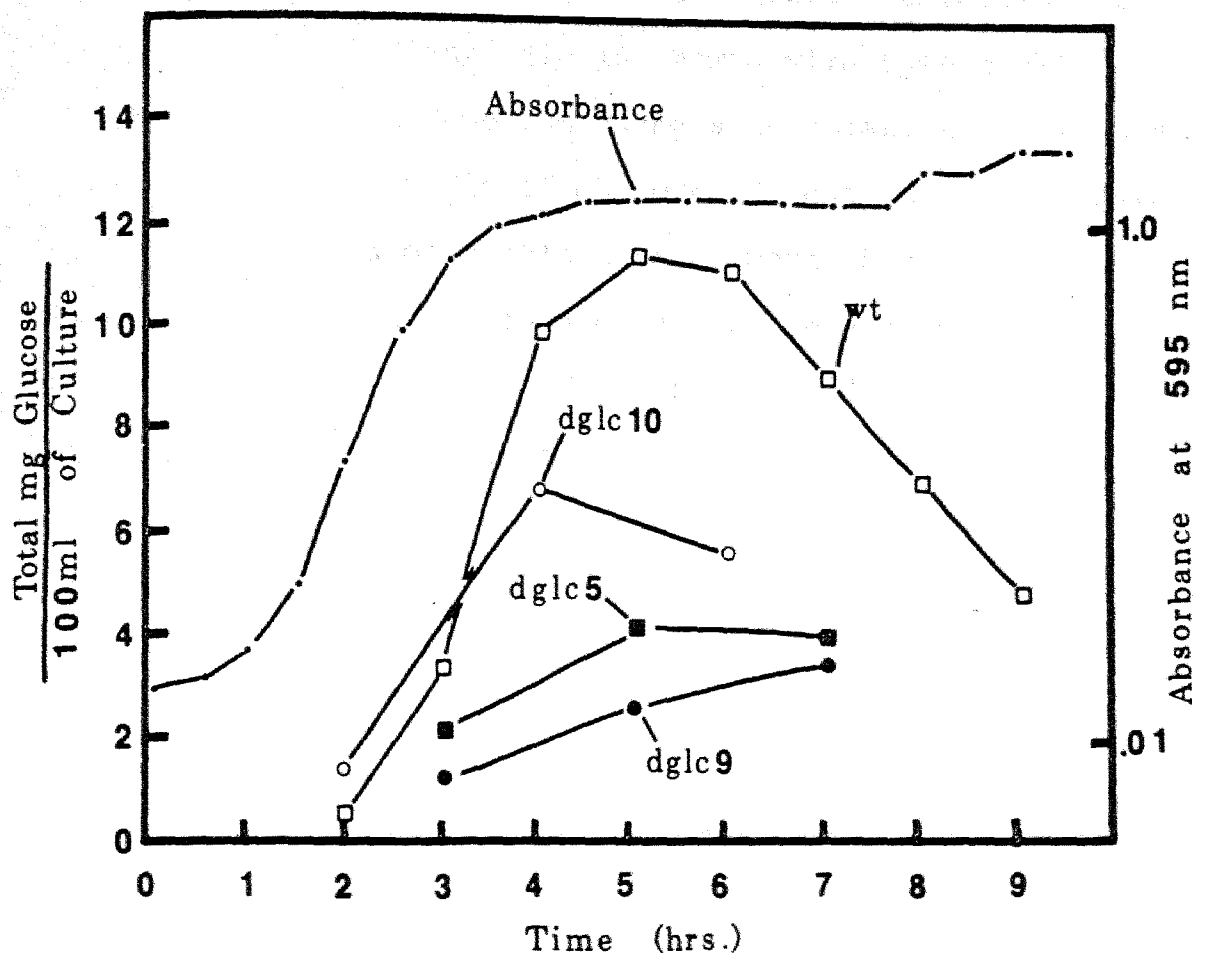

Figure 3. Total glucose of mutants in weakly buffered N medium. Mutant types 5, 9, and 10 were grown in weakly buffered N medium as described in materials and methods. Total glucose was measured with glucostat reagent with procedures found in materials and methods. Total glucose content of wild type cells in weakly buffered N medium are shown for comparison.

spores were produced which made this strain unsuitable for further study. Mutant dglc 10 showed wild type growth characteristics in G medium along with relatively low total glucose levels. Levels of glucose content increased sharply in N medium which made this strain unsuitable for further study. Low levels of glucose content were seen in both G and N medium for dglc 5. This strain produced a large amount of mature spores and had morphology similar to wild type during various stages of growth. On the basis of the properties examined, dglc 5 was used for further study to determine a possible function of intracellular polysaccharide during sporulation.

Poly-β-hydroxybutyric acid levels of wild type and dglc 5 cells. Both wild type and dglc 5 cells were grown in weakly buffered G medium and G medium buffered at pH 7.4. Samples were taken at various stages of growth for PHB determination as described in materials and methods. Accumulation and utilization of PHB in normal weakly buffered G medium is illustrated in Figure 4. Accumulation of PHB in wild type cells was similar to descriptions in other studies (Slock, 1970; Slepecky and Law, 1961). Strain dglc 5 showed a large accumulation that peaks at least an hour before wild type cells. Both cell lines grew with normal characteristics of pH fluctuations. Determination of PHB in G medium buffered at pH 7.4 is illustrated in Figure 5. Both wild type and dglc 5 showed extremely

Figure 4

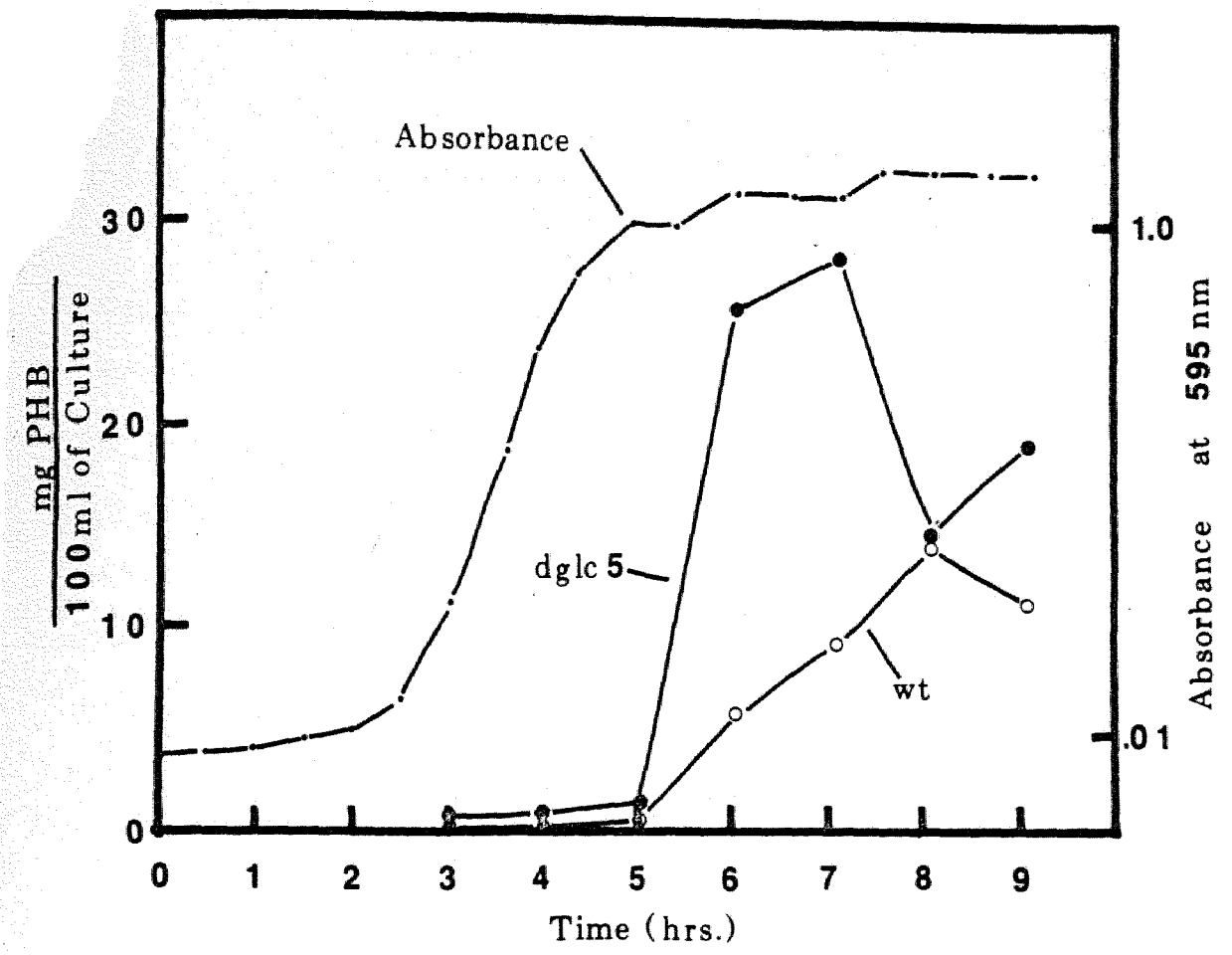

Figure 4. Poly-β-hydroxybutyric acid levels of wild type and dglc 5 cells in weakly buffered G medium. Cells of wt and dglc 5 were grown in weakly buffered G medium as described in materials and methods. Culture samples of 10 mls were measured for PHB content in cells with the procedure of Slepecky and Law as described in materials and methods.

Figure 5

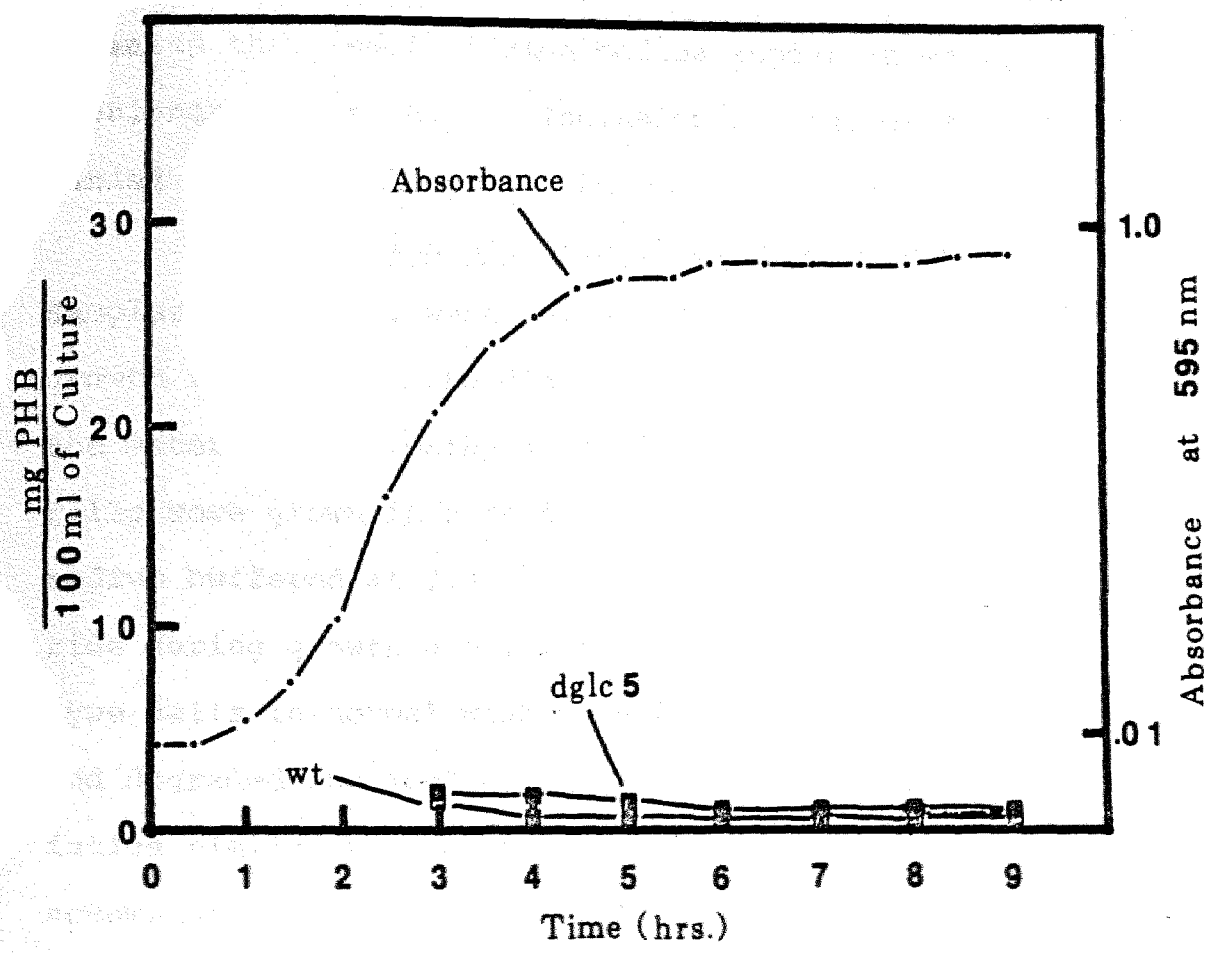

Figure 5. Poly-β-hydroxybutyric acid levels of wild type and dglc 5 cells in G medium buffered at pH 7.4. Cells were grown in G medium buffered at pH 7.4 and 10 ml culture samples were assayed for PHB content as shown in materials and methods.

low levels of PHB during growth. Microscopic examination revealed that few inclusion bodies appear in early stationary phase cells. Inclusion bodies normally accompanied the accumulation of PHB.

Determination of intracellular polysaccharide. Samples of 100 ml. were collected at various stages of growth followed by the disruption of cells as described in the materials and methods section. Wild type and dglc 5 cells were grown in normal weakly buffered G medium and G medium buffered at 7.4. Levels of intracellular polysaccharide during growth are illustrated in Figures 6 and 7. Wild type cells in normal weakly buffered G medium accumulated and degraded intracellular polysaccharide with characteristics similar to a previous study (Slock, 1970). Peak accumulations occurred at 3, 6, and 7 hrs. which correlated well with total glucose content levels (Figure 1) and the results obtained by Slock. The strain dglc 5 shows a definite deficiency in the ability to form the polysaccharide throughout the growth phases where accumulation takes place. The levels of intracellular polysaccharide in dglc 5 make up a fraction of total glucose content as shown in Figures 1 and 6. When wild type cells were grown in G medium buffered at 7.4 the wild type cells accumulated 1.8 mg/100 ml culture at 5 hr. when compared to 4.9 mg/100 ml culture at 7 hr. in weakly buffered G medium (Figure 7). The strain dglc 5 showed no sign of accumulation of intracellular

Figure 6

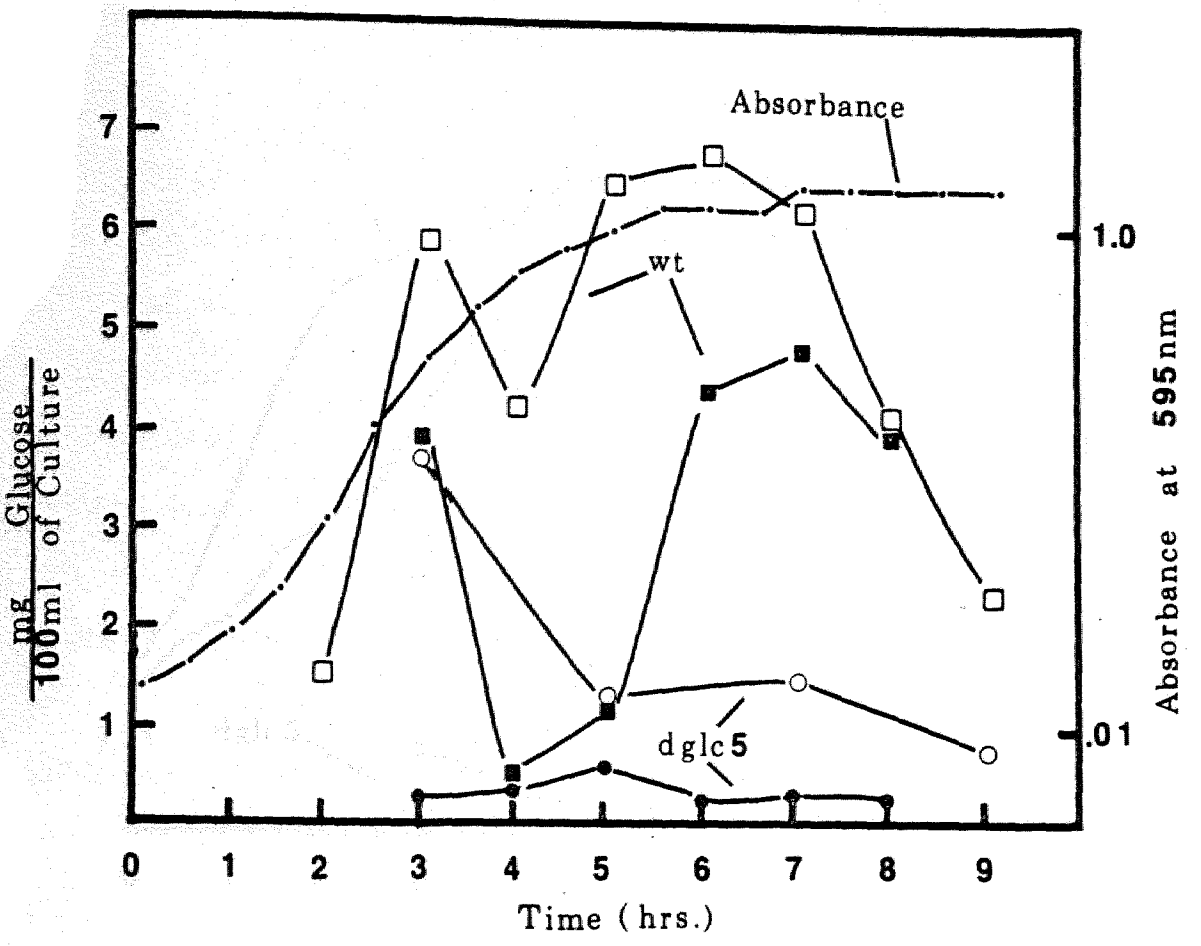

Figure 6. Intracellular polysaccharide content and total glucose content of wild type and dglc 5 cells grown in weakly buffered G medium. Levels of intracellular polysaccharide (expressed as mg glucose) in wt (■) and dglc 5 (●) were compared with total glucose content of wt (□) and dglc 5 (○) cells.

Figure 7

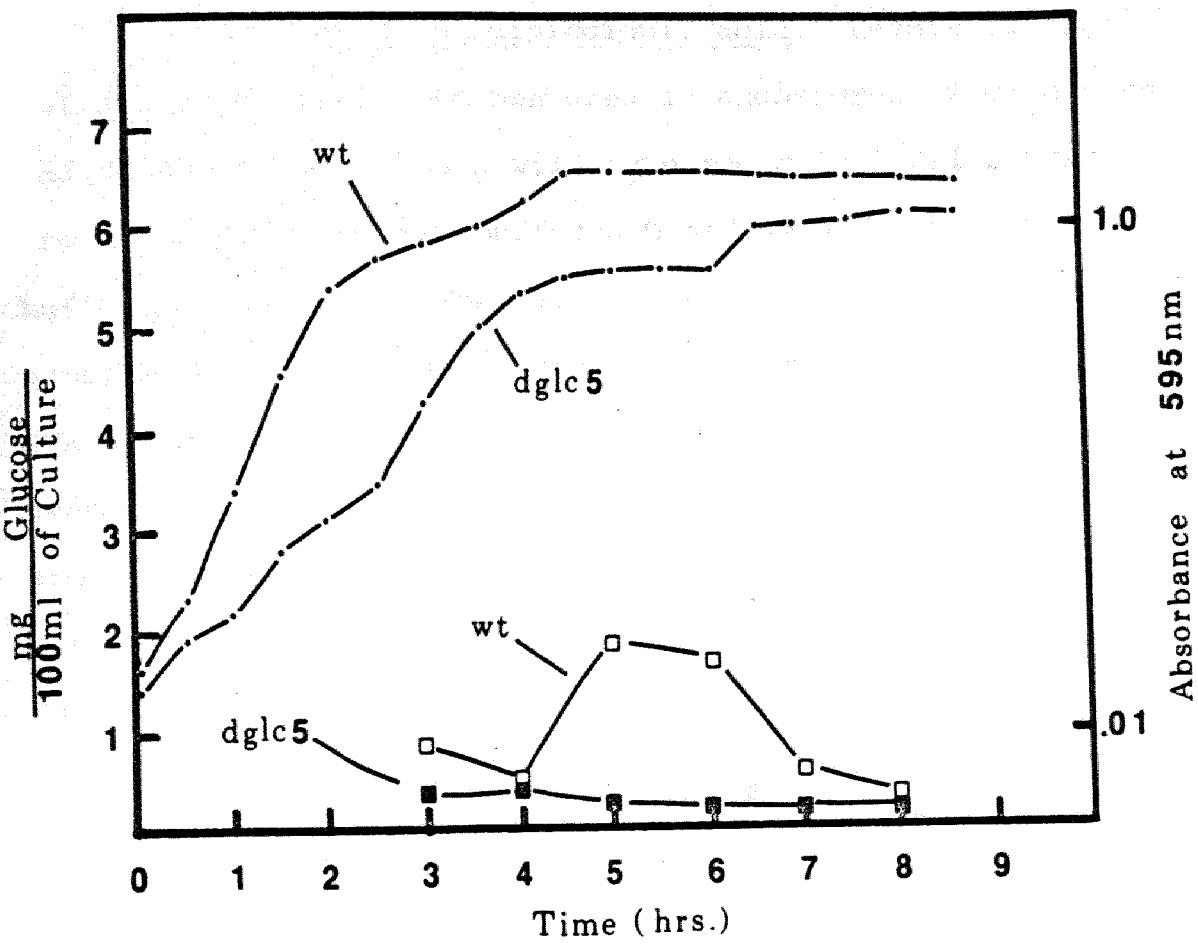

Figure 7. Intracellular polysaccharide content of wild type and dglc 5 cells in G medium buffered at pH 7.4. Cells were grown in G medium buffered at pH 7.4 and assayed with glucostat special for levels of intracellular polysaccharide as described in materials and methods. Intracellular polysaccharide content is expressed in mg glucose for wt (□) and dglc 5 (■) cells.

polysaccharide when grown in G medium buffered at 7.4.

Determination of dipicolonic acid. Levels of dipicolinic acid (DPA) were measured in endospores of wild type and dglc 5 origin. Both wild type and dglc 5 cells were grown in normal weakly buffered G medium and G medium buffered at pH 7.4. The cells were allowed to sporulate and samples were collected 48 hrs. after the start of log phase. DPA determination was done with modified Janssen reagent as described in the materials and methods section. DPA levels are illustrated in Table 2. When levels were examined in terms of µg/spore DPA remained constant in wild type and dglc 5 in both types of media. These levels compared with other findings quite well (Janssen et al., 1958).

Endotrophic sporulation with glucose addition. Cells of both strains were grown in weakly buffered G medium and G medium buffered at pH 7.4. At a selected time in early stationary phase samples were taken and a study of endotrophic sporulation was performed as described in the materials and methods section. Each sample was split and allowed to sporulate in endotrophic sporulation medium with and without the addition of glucose. The effects of glucose addition are shown on Figure 8. All samples were inhibited from sporulating by the presence of glucose. Strain dglc 5 shows greater inhibition than wild type in weakly buffered G medium. Under conditions of depressed PHB levels the heat resistance of wild type became similar to dglc 5. The

TABLE 2

DIPICOLINIC ACID LEVELS IN WILD TYPE AND MUTANT SPORES

Spore Strain	Medium	DPA µg/spore
Wild Type	Normal G medium	8.6×10^{-8}
		6.2×10^{-8}
	G medium buffered at pH 7.4	9.4×10^{-8}
		4.2×10^{-8}
dglc 5	Normal G medium	1.5×10^{-7}
		1.1×10^{-7}
	G medium buffered at pH 7.4	2.7×10^{-8}
		8.7×10^{-8}

Figure 8

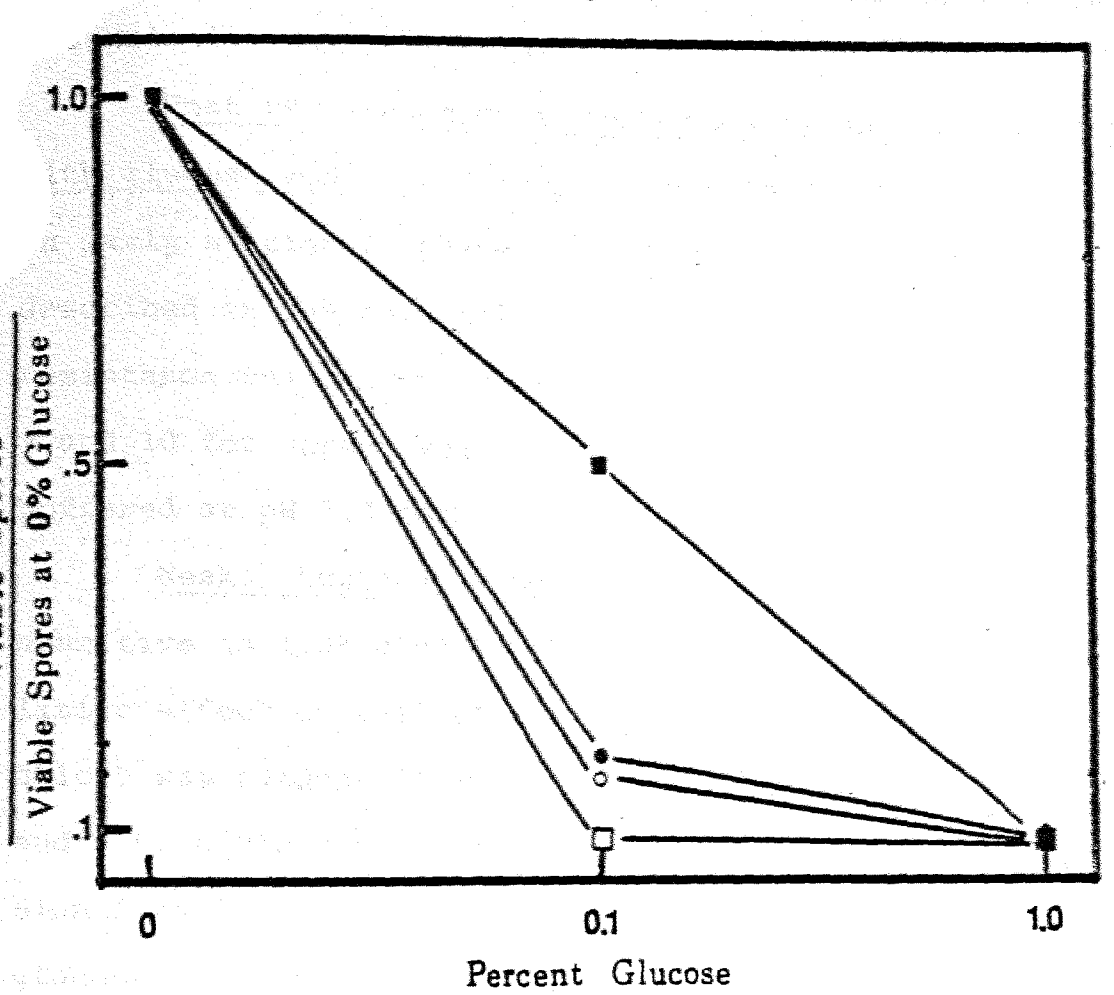

Figure 8. Viable spore yields of wild type and dglc 5 subjected to endotrophic sporulation with glucose addition. Wild type and dglc 5 cells were grown in weakly buffered G medium and G medium buffered at pH 7.4 as described in materials and methods. Samples of cells were taken at 4 and 5 hours of growth for wild type and dglc 5 respectively. The samples were subjected to endotrophic sporulation with glucose addition as shown in materials and methods. Viability of spores is represented with wild type grown in weakly buffered G medium (■), and G medium buffered at pH 7.4 (●). Mutant strain dglc 5 is represented in weakly buffered G medium (□) and G medium buffered at pH 7.4 (○).

mutant strain showed little change in resistance when PHB accumulation was inhibited.

Heat resistance of endotrophically sporulated samples with glucose addition. Samples were taken at a selected time in early stationary phase and subjected to the procedures described in the materials and methods section. Heat resistance results are shown on Tables 3 and 4 and Figures 9 and 10 for normal weakly buffered G medium and G medium buffered at pH 7.4 respectively.

Weakly buffered G medium. Wild type spores were heat sensitive to 1.0% glucose treatment while 0.1% glucose had little effect on resistance. The low heat resistance of dglc 5 was evident in spores lacking the glucose addition and a 0.1% glucose treatment did not change this resistance significantly. The heat resistance of dglc 5 drops when the glucose concentration was increased to 1.0%. Of all the curves shown, only wild type and dglc 5 spores that are lacking glucose treatment showed the characteristic enhancement of germination from heat shock.

G medium buffered at pH 7.4. Wild type spores showed good resistance to heat at 1.0% and 0% glucose treatment levels. Some sensitivity was shown at 0.1% glucose level. The heat resistance for dglc 5 spores lacking glucose addition was similar to dglc 5 spores without glucose addition in normal weakly buffered G medium which allowed PHB accumulation. In wild type and dglc 5 spores the addition

TABLE 3

HEAT RESISTANCE OF WILD TYPE AND dglc 5 GROWN IN WEAKLY BUFFERED G MEDIUM AND SUBJECTED TO ENDOTROPHIC SPORULATION WITH GLUCOSE ADDITION

		Time (mins) at 80°C											
		0		5		10		20		30		60	
	% glucose		std dev		std dev		std dev		std dev		std dev		std dev
Wild Type	0.0	100	0	110	14	108	19	85	7	84	2	96	15
	0.1	100	0	86	7	95	5	100	14	93	6	89	11
	1.0	100	0	85	12	71	14	58	4	57	10	52	5
dglc 5	0.0	100	0	83	8	65	6	55	5	53	11	27	2
	0.1	100	0	106	8	97	5	78	11	51	3	40	6
	1.0	100	0	10	2	8	7	1	1	0	0	0	0

Values presented as % survival.

TABLE 4

HEAT RESISTANCE OF WILD TYPE AND dglc 5 GROWN IN G MEDIUM BUFFERED AT pH 7.4

		Time (mins) at 80°C											
		0		5		10		20		30		60	
	% glucose		std dev		std dev		std dev		std dev		std dev		std dev
Wild Type	0.0	100	0	93	7	92	16	92	8	95	5	94	8
	0.1	100	5	79	11	80	5	91	12	81	16	69	9
	1.0	100	0	87	8	74	15	82	7	107	7	95	11
dglc 5	0.0	100	0	83	9	83	10	53	4	85	21	42	3
	0.1	100	0	95	12	75	5	81	12	73	7	84	6
	1.0	100	0	100	5	100	14	80	5	100	21	50	7

Values presented as % survival.

Figure 9

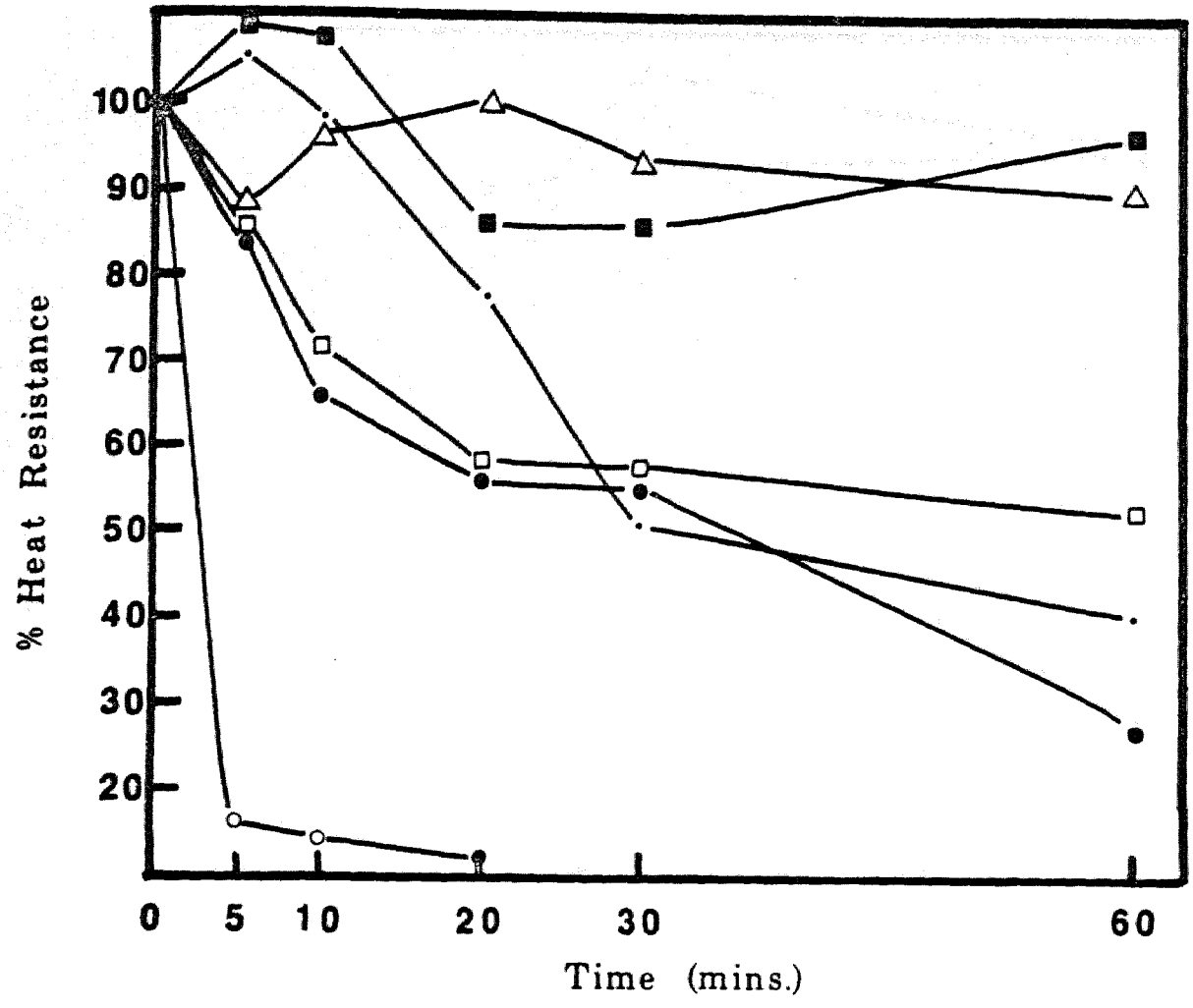

Figure 9. Heat resistance of wild type and dglc 5 grown in weakly buffered G medium and subjected to endotrophic sporulation with glucose addition. Cells were grown in weakly buffered G medium and sampled at 4 and 5 hours for wild type and dglc 5 respectively. The samples were placed in endotrophic sporulation media with 0, 0.1 and 1.0% glucose and allowed to sporulate. Heat resistance was determined by exposure of spores to 80°C as described in materials and methods. Percentage heat resistance of spores is represented in wild type for 0% glucose (■), 0.1% glucose (△), and 1.0% glucose (□). Percentage heat resistance in dglc 5 spores is represented for 0% glucose (●), 0.1% glucose (·), and 1.0% glucose (○).

Figure 10

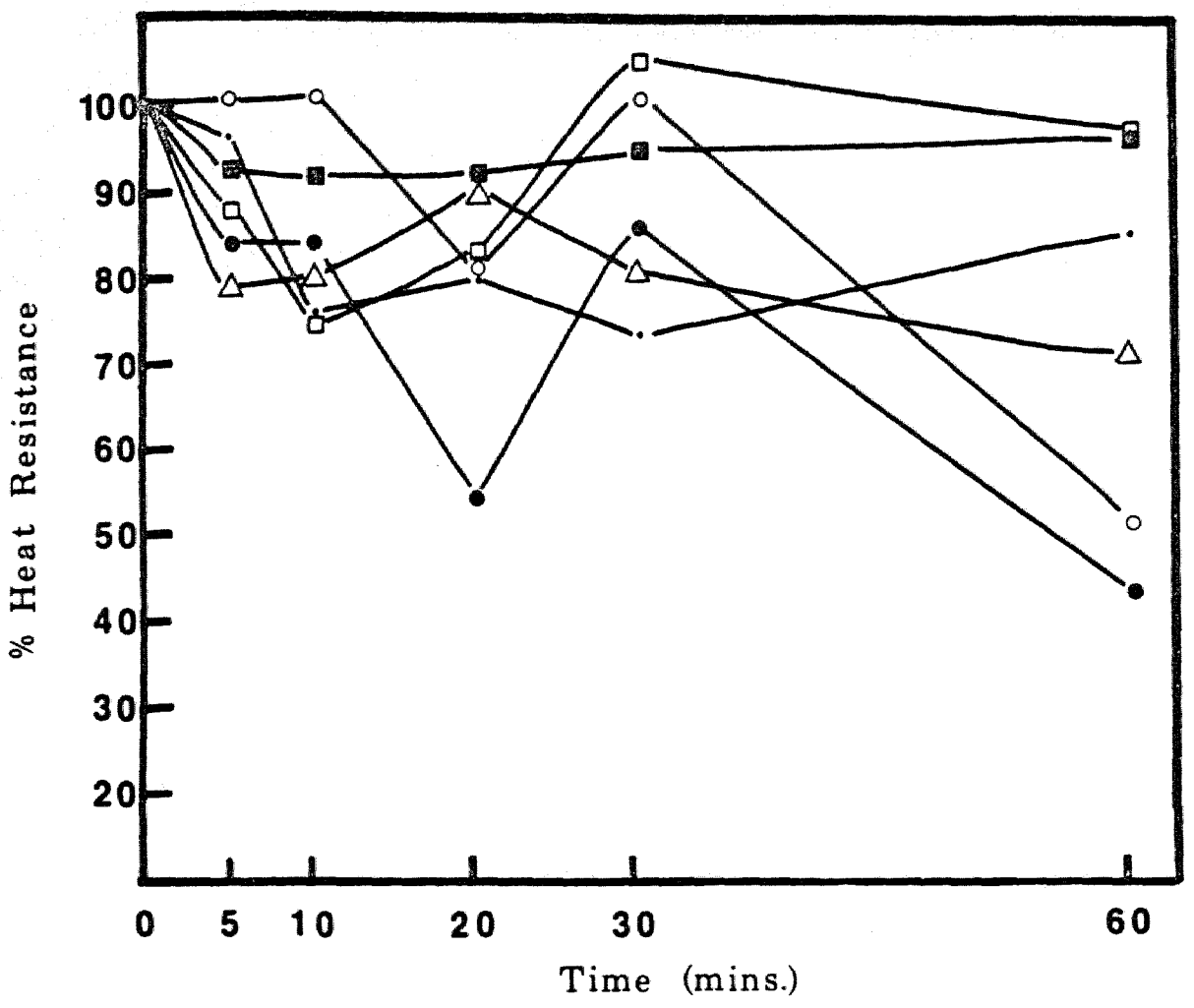

Figure 10. Heat resistance of wild type and dglc 5 grown in G medium buffered at pH 7.4. Cells were grown in G medium buffered at pH 7.4. Samples were taken at 5.5 hours for both wild type and dglc 5. The samples were placed in endotrophic sporulation media with 0%, 0.1%, and 1.0% glucose and allowed to sporulate. Heat resistance was determined by exposure of spores to 80°C as described in materials and methods. Percentage heat resistance of spores is represented in wild type for 0% glucose (■), 0.1% glucose (△), and 1.0% glucose (□). Percentage heat resistance in dglc 5 spores is represented for 0% glucose (●), 0.1% glucose (·), and 1.0% glucose (○).

of glucose inhibited heat resistance when PHB was accumulated and enhanced resistance when PHB accumulation was depressed.

DISCUSSION

Comparison of wild type and dglc 5 cells during growth revealed similar characteristics. Changes in morphology of dglc 5 during the logarithmic, stationary, and sporulation phases appeared similar to wild type when grown under the same conditions. Vacuole formation and the appearance of forespore bodies occurred at approximately the same time of growth in the mutant and wild type. The mutant consistently produced a longer log phase than wild type but the rates of growth during the logarithmic phase were equal. Wild type and dglc 5 developed refractility of the forespores and then proceeded to form mature spores. Under examination with phase contrast microscopy the mutant strain displayed no visible defects in growth and sporulation when compared to the wild type under the same conditions.

The biochemical characteristics of mutant strain dglc 5 suggests an area of metabolism where mutation has occurred. Wild type and dglc 5 cells grown under similar conditions exhibited the same fluctuations of pH in the media. When grown in G medium a pH drop indicated exhaustion of exogenous glucose and a build up of organic acid products from glucose metabolism (Goldman and Blumenthal, 1964). As the pH began to increase, acetate was oxidized

to CO_2 and PHB levels increased (Hanson et al., 1963). During the pH rise cells in the vegetative state entered the initial stages of the sporulating process. From these results it was assumed that no major defects were present in the mutant strain which involved glucose catabolism or oxidation of organic acids. The large accumulation of PHB in dglc 5 cells may be indicative of a mutation or a condition caused by the cell's inability to accumulate intracellular polysaccharide. The inability to accumulate intracellular polysaccharide was clearly shown in Figures 6 and 7. Blockage of the gluconeogenesis pathway or polysaccharide formation was evident by the depressed levels shown in dglc 5.

DPA levels of wild type and dglc 5 spores remained constant under conditions favorable for PHB accumulation and conditions that repressed accumulation. The mutant spore DPA content was within the normal limits found in wild type spores. DPA has been closely linked with the acquisition of heat resistance as with the uptake of calcium. The lack of intracellular polysaccharide and/or PHB did not restrict the formation of DPA in spores as seen in Table 2.

The endotrophic sporulation of wild type and dglc 5 cells with the addition of glucose gave results consistent with a similar study (Strasdine, 1972). Exogenous glucose inhibits the formation of spores and had various effects on the spore's ability to acquire heat resistance. Wild type

cells grown in G medium that undergo sporulation in 1.0% glucose, 0.008% $CaCl_2$ solution became heat sensitive. Mutant strain spores were not resistant to heat and the resistance sensitivity decreased when glucose was added to endotrophic sporulation media as seen in Figure 9. Lowered heat resistance is seen in cells which were allowed to accumulate PHB. Previous work indicates that conditions favorable for PHB production resulted in a lower yield of mature spores (Slepecky and Law, 1961). Upon examination of Figures 9 and 10 there appeared to be a relation between PHB levels, exogenous glucose levels, and the resulting heat resistance of spores. Figure 10 revealed that dglc 5 was more resistant to heat when PHB levels were low and glucose was added to endotrophic sporulation media. A specific amount of exogenous glucose was needed for optimal heat resistance in dglc 5. When PHB has accumulated and cells were exposed to low levels of glucose dglc 5 showed increased heat resistance. Higher levels of glucose addition decreased the heat resistance dramatically. Because low levels of glucose could be beneficial to the heat resistance qualities of dglc 5 spores, intracellular polysaccharide appeared to be an endogenous source of carbon and energy for the sporulation process of Bacillus cereus.

CONCLUSIONS

A mutant strain of Bacillus cereus has been isolated which lacks the ability to accumulate intracellular polysaccharide. The mutant demonstrated normal growth characteristics but produced a low level of spores which are sensitive to heat. This sensitivity can be increased when the mutant was grown in conditions favorable for PHB accumulation and allowed to sporulate in media containing 0.008% $CaCl_2$ and various amounts of glucose. Heat resistance can be enhanced in mutant spores with growth conditions which inhibit PHB formation followed by sporulation in a solution of 0.008% $CaCl_2$ and 0.1% glucose. By comparison of these properties with wild type spores, intracellular polysaccharide can be considered a survival factor in the formation of mature spores of Bacillus cereus.

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